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ROY WALDO MINER

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BY

ARTHUR J. KAHN AND ALEXANDER SANDOW

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Awarded Honorable Mention in Competition for an A. Cressy Morrison Prize in Natural Science in 1954 by The New York Academy of Sciences

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EFFECTS OF BROMIDE, NITRATE, AND IODIDE ON RESPONSES OF SKELETAL MUSCLE*

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A series of investigations on the effects of the potassium ion upon the mechanical and electrical responses of amphibian skeletal muscle was carried out by the writers from 1949 to 1950.[1,2,3] Inasmuch as potassium had been introduced as KCl in these experiments, a control series was undertaken using K_2SO_4 and KNO_3 and, while the use of these latter salts definitely showed that the observed results were due to K rather than Cl, the nitrate-treated muscles showed an additional effect, a large potentiation of the maximal twitch response, not obtainable with either KCl or K_2SO_4.

It has been known for some time that nitrate and other anions of the lyotropic series are able to act upon frog skeletal muscle to cause an increase in the height of contraction obtained by various stimulating agents.[4-12] These investigations led to the general view that the anions increased the contractile response of skeletal muscle by a "sensitizing" action upon the excitatory membrane of the individual muscle fibers. Potentiation of the contractile response was thus attributed to a heightened excitability of the muscle.

Of these earlier anion studies, only the investigations of Schwarz[4] and of Chao[11 and 12] employed electrical stimulation of the tissue and, since submaximal intensities of stimulation were used, this permitted drawing the conclusion that the potentiation of the mechanical responses was due to recruitment which appeared in consequence of increased excitability of previously unresponsive fibers. The preliminary nitrate studies conducted in this laboratory,[13] however, employed electrical stimuli which were of slightly supermaximal strength, thus insuring responses of all of the fibers of the muscle prior to the experimental treatment, and since the increased twitch tension outputs were still observed in the nitrate medium, they could not be attributed to a recruitment of additional fibers but appeared to be the result of a definite increase in the mechanical output of each of the fibers of the muscle. In

*These studies were aided by a contract (NR 113-300) between the Office of Naval Research, United States Department of the Navy, Washington, D. C., and New York University, New York, N. Y. Preliminary notices of some of this work have appeared as follows: in *Science*, **112**: 6478- 649 (1950); *Biol. Bull.* **99**: 316-317 (1950); and *Fed. Proc.*, **10**(1): 71 (1951).

**This paper is taken from a thesis submitted by Doctor Kahn to the Faculty of the Graduate School of Arts and Science, New York University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

elaboration of this finding, this paper presents the results of a detailed investigation of the effects of nitrate, and of bromide and iodide as well, on amphibian skeletal muscle as they relate to certain fundamental problems in excitation, contraction, and relaxation, and in the interconnecting links between these events.

General Procedures

The muscles studied (sartorii of the frog, *Rana pipiens*) were each excised with pelvis attached and, in all instances, were preliminarily equilibrated for one hour in oxygenated standard (chloride–) Ringer's solution (NaCl, 0.115M; $CaCl_2$, 0.0018M; KCl, 0.002M; buffered with 4mM phosphate to pH 7.2). From three to five muscles were used in every procedure for each experimental or control solution, and all experiments were done at 25° C. Isometric mechanical responses of sartorii were recorded by means of the piezoelectric, cathode-ray oscillographic method, and the initial and peak developed tensions were recorded by simultaneous optical myography.[14] The electrical equivalent of the mechanical output was fed into an electronic differentiating circuit with a time constant of 4×10^{-5} second. This was accomplished by taking advantage of the fact that the piezoelectric unit we used (the Astatic phonograph pickup cartridge L-72A), in addition to acting as a voltage generating mechanoelectric transducer, had an internal series capacitance of about 0.002 μ). Connection of the unit to a grid resistor of 20 K ohms at the input of our amplifier thus provided the differentiating circuit with the aforementioned time constant. This circuit could not differentiate any arbitrarily chosen pulse, but it was designed to do this for the tension changes characteristic of the contractions encountered in our experiments. The critical need was to make the circuit capable of adequately differentiating the fastest rate of change, such as occurs during the contraction period. An approximate measure of this requirement is given by the duration of the contraction period, about 30 msec. at the temperature (25° C.) of our experiments. That our differentiating circuit satisfied this requirement is indicated by the fact that, when sine waves of constant voltage were fed into the circuit, the output voltage was a perfectly linear function of the frequency up to at least 400 cycles/sec. Furthermore, a more direct test was made by comparing the derivative of a given twitch obtained electronically with that determined graphically from the direct tension record registered simultaneously by optical myography. The two derivative curves agreed, on the average, to within 2 to 3 per cent (the peak rate of tension change during the contraction period being set at 100 per cent for each curve), and this deviation was in all probability due chiefly to unavoidable error in making the graphical derivative.

Thus this circuit was capable of differentiating, with respect to me, the contractile phase and the postcontractile relaxation phase of ne electrical equivalent of the mechanical response, *i.e.*, the tension hanges undergone by the muscles were recordable, through the use of he differentiating circuit, as the rates of change of tension (d/dt tension) vithin the duration of the mechanical response.[15] The precontractile atency relaxation, however, was rapid enough to permit the latent period events to pass through this circuit with only a small degree of differntiation. This very slight alteration was compensated for by an integrating circuit in a later stage of amplification. After passing through he differentiating circuit, the mechanical responses were, in effect, separated into two components (the essentially unaltered mechanical latent period events and the differentiated mechanical responses) each of which was separately amplified and separately but simultaneously visualized on a Dumont Dual Beam Cathode-Ray Oscillograph. The amplifier for the latency changes was of an ordinary R-C coupled type with a time constant of 0.5 sec. and linear to about 6000 cycles/sec.; and that for the twitch-derivative was, for most of the experiments, a 20 sec. time-constant, R-C amplifier (the long time-constant being required to amplify without distortion the relatively slow twitch changes), and it was linear to about 4000 cycles/sec. In some of the later experiments, the derivative amplifier was D-C coupled and good to about 12,000 cycles/sec.

The muscle chamber was of a design that permitted stimulation by means of massive transverse shocks. This method was first used for single muscle fibers by Brown and Sichel[16] and was later developed for use on a whole muscle by Sandow.[17] It has been shown[17] that this type of stimulation produces a more synchronous response than that obtained when the conventional wire electrode type of stimulation is employed. Massive stimulation was used to greatest advantage wherein it was desired to know the effect of a particular anion as soon as possible after contact with the muscle and without interruption thereafter during the continued action of the anion.

In all experiments, with the exception of the strength-duration and latent addition studies, stimulation was accomplished through the use of condenser discharges obtained by conventional methods from a thyratron stimulator. The time constant for these stimuli was 0.2 msec. For the other types of studies, rectangular-wave shocks were employed. These latter stimuli were obtained from a new high-current, rectangular-wave stimulator.[18] For the recording of action potentials of sartorii, the muscles were temporarily removed from their solution and, while attached to the isometric lever, were supported in a moist (wire electrode) chamber. Conventional external electrodes and recording methods were employed,

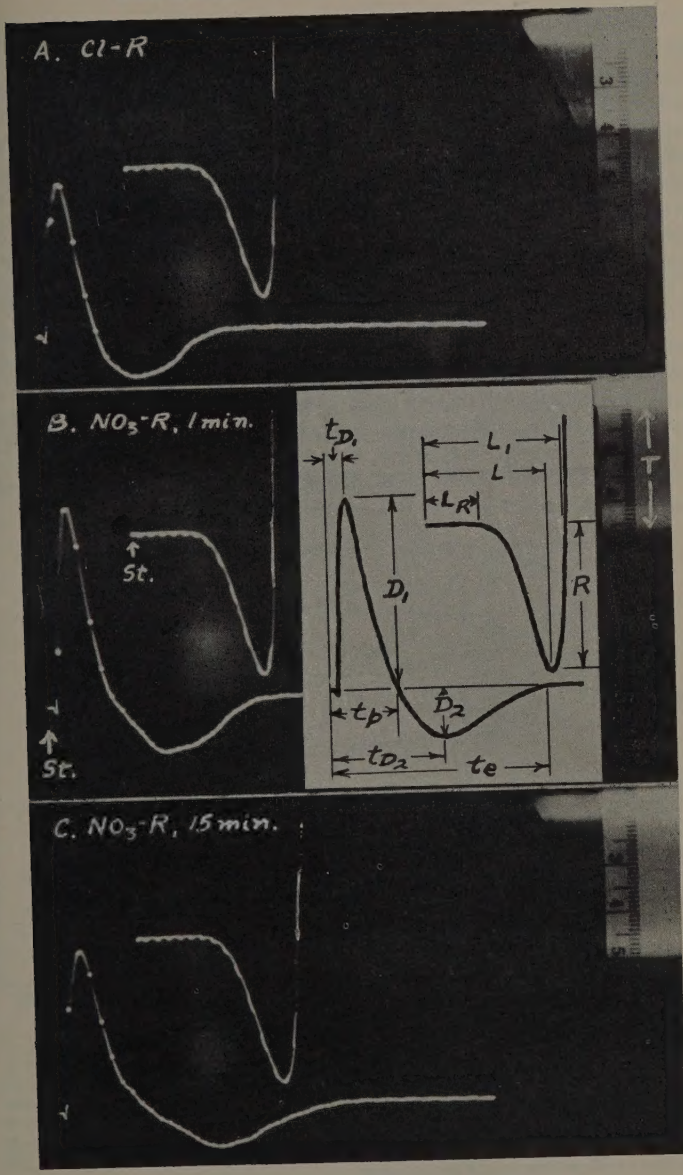
and the stimuli were applied to the pelvic, nerve-free portion of the muscles.

The experimental solutions were in all instances isotonic to the standard (chloride–) Ringer and contained cations in identical ratios. They differed only in that the chloride was totally absent and was replaced with one of the experimental anions (bromide, nitrate, or iodide). These experimental solutions, which we name, respectively, bromide-Ringer, nitrate-Ringer and iodide-Ringer, were buffered in the same manner and to the same pH as was the control chloride-Ringer) solution. Three grams of initial tension was applied to the muscles and maintained throughout the duration of all experiments.

Results

(1) *Isometric twitch responses of sartorii treated with either bromide-Ringer, nitrate-Ringer, or iodide-Ringer.* Following equilibration, sartorii were mounted in the massive electrode chamber in chloride-Ringer, the maximal shock strength was determined for each, and the stimulus intensity was adjusted to a slightly supermaximal value. Two normal maximal twitch responses separated by a two-minute interval were obtained. Immediately thereafter the chloride-Ringer was withdrawn, and the chamber was filled with one of the three experimental solutions or with chloride-Ringer, and isometric twitch responses (to the same previously used, slightly supermaximal stimuli) were obtained at 1, 3, 5, 13, 27, 39, 51, 75, 105, and 120 minutes. At 120 minutes, the experimental solution was replaced with chloride-Ringer and the responses of the muscle to the same stimuli were recorded at 1, 3, 5, 10, 15, 30, and 60 minutes.

FIGURE 1. Typical records showing various parameters of the isometric twitch of a frog sartorius muscle, and effects of the NO_3 ion on these, at 25° C. Initial tension, 3 gm. Each record includes the following (see inset of 1B for diagrammatic representation of the different parameters): at the *upper right corner*, the upper position of the light beam indicates the resting tension of the muscle; and the lower position, the deflection corresponding to peak deflection corresponding to peak developed tension, T. The *upper oscillographic trace*, impressed with an intensity modulated timing-wave at 5000 cycles/second, gives the direct changes of the latent period with the following durations all measured from the instant of stimulation (St): L_R, to the beginning of the latency relaxation; L, to the end of the latency relaxation; L_1, to the onset of tension above the initial tension. R measures the depth of the latency relaxation. The *lower oscillographic trace*, intensity modulated at 200 cycles/second, gives the time-derivative (*i.e.*, the rate of change of tension) of the twitch and it includes the following durations all measured from the instant of stimulation: t_{D_1}, to the moment of greatest rate of tension increase in the rising phase of the twitch; t_p, to peak of twitch; t_{D_2}, to the moment of greatest rate of tension decrease in the falling phase of the twitch; and t_e, to the end of the twitch. D_1 measures the maximum rate of rise of tension, and D_2, the maximum rate of fall of tension. The records show the various parameters of a twitch of a single muscle; (A) in Cl-Ringer, and then after immersion in NO_3-Ringer, (B) for 1 minute and (C) for 15 minutes. See text for further details.


A. Cl-R
B. NO3-R, 1 min.
St.
St.
tD1
L1
L
LR
D1
R
T
tp
D2
tD2
te
C. NO3-R, 15 min.

Typical results of this part of our study, which illustrate in general the kind of records we obtain by our method, are presented in FIGURE 1. All three records of this figure were obtained from a single sartorius, treated as indicated, and all amplifier, timing, and oscillographic controls were the same for all records. The record of FIGURE 1A gives the behavior of a muscle in Cl-Ringer. The muscle was then exposed to NO_3-Ringer and, one minute later, it produced the response given in FIGURE 1B, in which the following changes may be noted: an increase in developed peak tension (T) of 40 per cent; an increase in time to peak of twitch (t_p) of 30 per cent and to end of twitch (t_e) of 20 per cent; an increase in greatest rate of rise of tension during the contraction period (D_1) of 33 per cent and in greatest rate of fall of tension during the relaxation period (D_2) of about 15 per cent; an increase in depth of the latency relaxation (R) of about 7 per cent; and decreases in latent period time parameters (L_R, L, L_1) of the order of 0.1 msec. FIGURE 1C illustrates the behavior of the muscle after 15 minutes in the NO_3-Ringer, and it

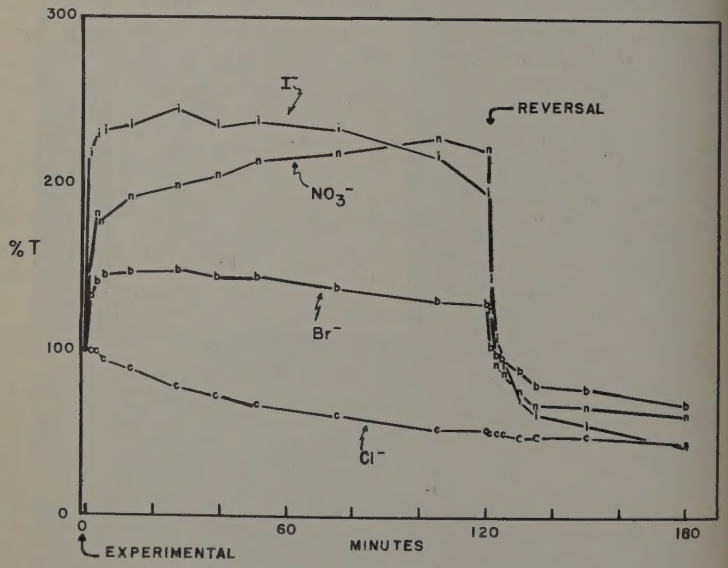


FIGURE 2. Average changes in maximal twitch tension of sartorii as a function of time in Cl-Ringer, and in Br^-, NO_3 , or I-Ringer. All values are plotted relative to those in Cl-Ringer just prior to the beginning of the experimental period. At 120 minute point, reversal was effected by removing the solution of the experimental period and replacing with Cl-Ringer. Average deviation of points on curve for the different Ringer solutions: Cl ± 4 per cent; Br, ± 8 per cent; NO_3, ± 26 per cent; I, ± 24 per cent.

an be easily seen especially that the developed peak tension is now till greater, by about 58 per cent of the original Cl-response, and the imes to twitch–peak and end of twitch are still further increased (e.g., e now is 65 per cent greater than it was in the Cl-medium). It is also pparent that the values of D_1 and D_2 have now decreased and are at bout their initial Cl-values. These and other changes, qualitatively, re essentially the same when the active ions are Br or I instead of NO_3. The following presents a detailed analysis of all these various effects.

One of the most striking of the changes due to the abnormal anions s the considerable potentiation of the maximal isometric twitch peak ension. The mean increases in twitch tension plotted as a function of he time in the experimental solution are presented in FIGURE 2. The 100 per cent level represents the peak isometric twitch tension developed by the muscles in chloride-Ringer and the experimental values are plotted as percentages of the pretreatment tensions. It is noteworthy that the maximal effects of the anions on the twitch tension output fall into a clear series, $Cl < Br < NO_3 < I$. It is also important to note the rapidity with which these anions were able to bring about these alterations; 60 to 80 per cent of the maximum potentiation brought about by bromide,

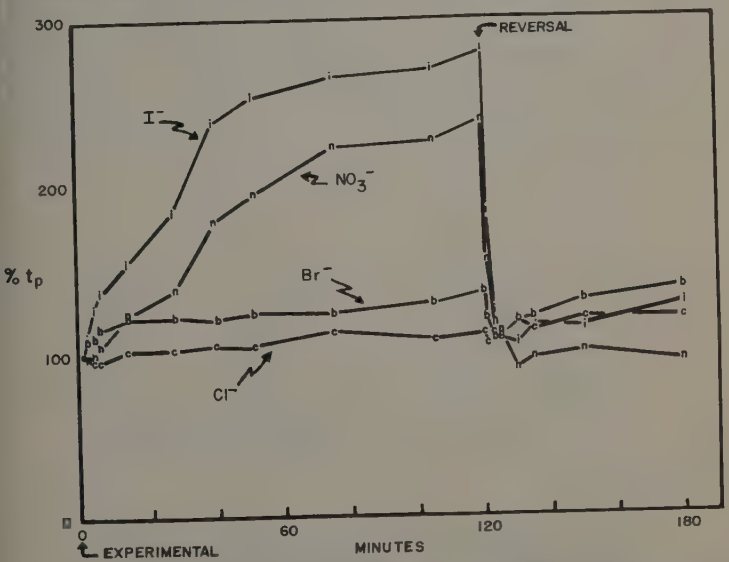


FIGURE 3. Effects of anions on the time to the peak of the twitch. Procedural details as in FIGURE 2. Average deviations of plotted points: Cl, ± 2 per cent; Br, ± 11 per cent; NO_3, ± 9 per cent; I, ± 23 per cent.

nitrate, and iodide occurred after only three minutes of contact with the muscles.

It is evident that the potentiated twitch tensions could not have resulted from a recruitment of additional fibers not previously responding, since the shock strength was maintained at the original slightly supermaximal value throughout the experiments. This magnitude of stimulus intensity insured that all of the fibers were responding, and that each muscle was performing a maximal response even in chloride-Ringer prior to treatment with the experimental anions. Hence, we conclude that, in each case, the observed potentiation of the response of a muscle was a consequence of an intrinsic potentiation of the response of each fiber of the muscle. The reversibility of these anionic effects is clearly demonstrated by the rapid return of the peak tension outputs of these muscles to nearly normal values within a relatively short period following the replacement of each experimental solution with chloride-Ringer.

The twitch derivative records for these same responses indicate changes in contraction time (t_p, FIGURE 3) and in total twitch time (t_e, FIGURE 4). Again, the values for the pretreated muscles in chloride-Ringer were set at the 100 per cent level and the average experimental values have been plotted as percentages of this normal level. It is apparent that the increases in maximum twitch tension brought about by the experimental anions are accompanied by increases in the contraction time, the relaxation time, and the total twitch time, and that the order of activity of these anions on t_p is (as it is on tension output)

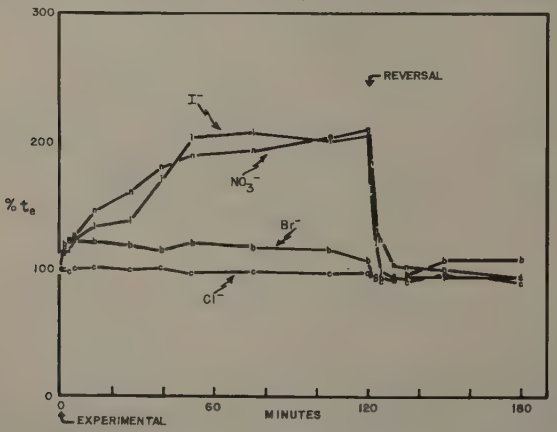


FIGURE 4. Effects of anions on the total twitch time. Procedural details as in FIGURE 2. Average deviations: Cl, ± 3 per cent; Br, ± 5 per cent; NO_3, ± 12 per cent; I, ± 4 per cent.

chloride < bromide < nitrate < iodide, while for t_e it is chloride < bromide < nitrate, iodide. The time from the instant of stimulation to the maximum rate of positive tension development (t_{D_1}) was not altered by the experimental treatment, but the corresponding time to the moment of maximum rate of relaxation (t_{D_2}) was increased (FIGURE 5). The derivative records indicate also that in these same responses the maximum rate of tension change in contraction (D_1) (FIGURE 6) and in relaxation (D_2) (FIGURE 7) were increased considerably and rapidly by these anions although, as demonstrated in FIGURE 1, a partial return toward the normal level was always observed with continued anion action. In general, all of these effects on parameters of the twitch derivative were quickly reversed following replacement of the treated muscles in Cl-Ringer's solution.

Important changes were also noted in the latent period responses of treated muscle, although the effects vary with time of exposure to the experimental anions in a generally more complex way than for those of most of the previously described changes. In any case, it should be noted, first, that the entire latent period in our experiments is of the order of only two to three msec. Nevertheless, the reliability of behavior of the muscles and the precision of the electronic method for registering the latency mechanical changes is such (see Sandow[14] and note the consistency of the present results) that changes in the duration of latency parameters of the order of ± 0.05 msec. may be considered significant.

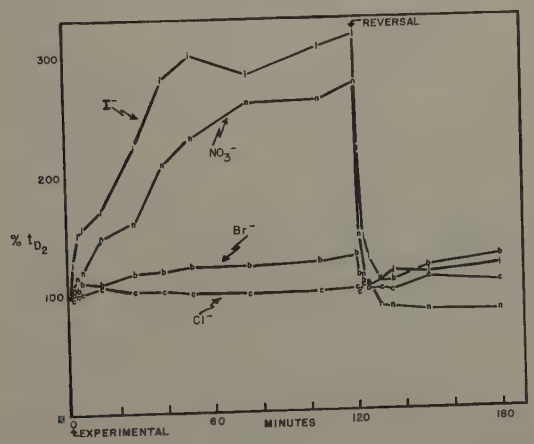


FIGURE 5. Effects of anions on the time to moment of maximal rate of tension fall in the relaxation period. Procedural details as in FIGURE 2. Average deviations: Cl, ± 5 per cent; Br, ± 6 per cent; NO_3, ± 16 per cent; I, ± 10 per cent.

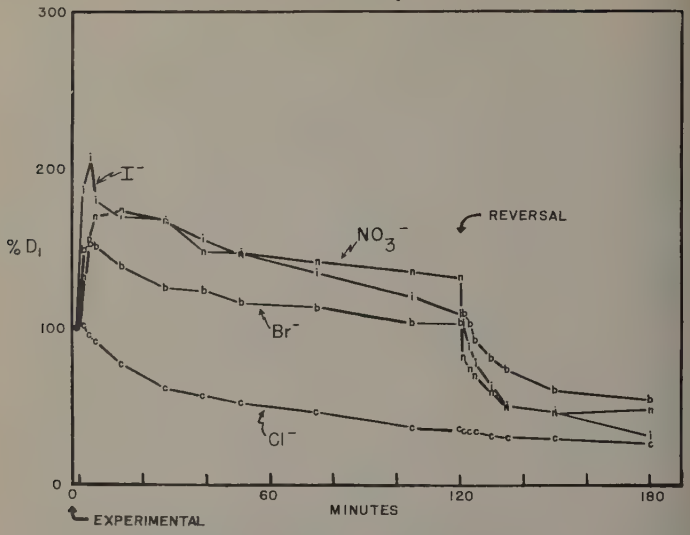


FIGURE 6. Effects of anions on the maximal rate of tension rise during the contraction period. Procedural details as in FIGURE 2. Average deviations: Cl, ± 3 per cent; Br, ± 12 per cent; NO_3, ± 23 per cent; I, ± 19 per cent.

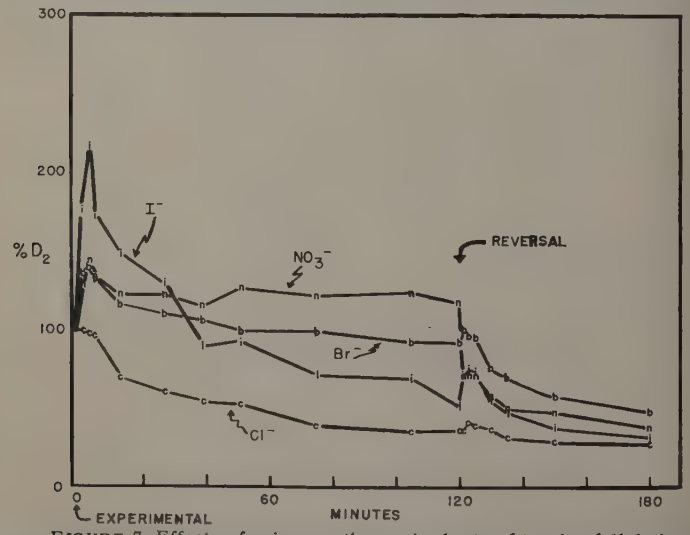


FIGURE 7. Effects of anions on the maximal rate of tension fall during the relaxation period. Procedural details as in FIGURE 2. Average deviations: Cl, ± 2 per cent; Br, ± 3 per cent; NO_3, ± 14 per cent; I, ± 16 per cent.

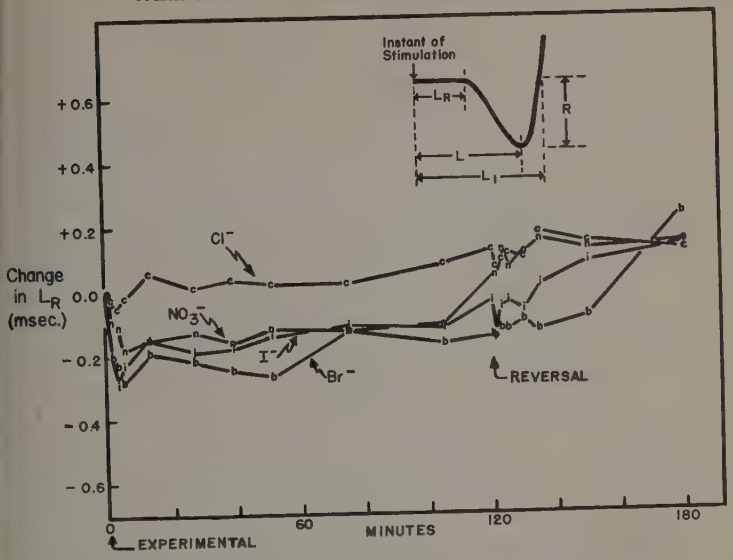


FIGURE 8. Effects of anions on the time to the onset of the latency relaxation. Procedural details as in FIGURE 2. Average deviation for all points, ± 0.06 msec.

FIGURE 8 shows that the value of L_R is quickly reduced by about 0.2 msec. by action of each of the experimental anions and that, after a fairly constant maintenance of this reduction for almost two hours, it is then reversed when the muscles are again restored to the chloride-Ringer's. The effects on L (FIGURE 9) and L_1 (FIGURE 10) are like those on L_R, though more pronounced in respect to the rapidly produced initial decreases. Then, however, these decreases tend to be reversed, even though the involved muscles are still in their experimental media (a behavior like that previously encountered with respect to D_1 and D_2), and then, upon return to Cl-Ringer's, all the previously treated muscles, except those in the NO_3 medium, passed through a cycle of alterations rather like those they experienced when first exposed to their respective test media. In all of these results, any order of effects in accord with a lyotropic series is highly blurred or absent. Yet it is noteworthy that the experimental anions had in common the general effect of reducing the parameters of the latent period in comparison with the corresponding values for the Cl-muscles. It is also of interest, however, that, in detail, the course of these reductions with time of immersion in the anionically modified media is different for the interval L_R from that observed for L and L_1. A similar distinction in behavior of L_R, which measures the

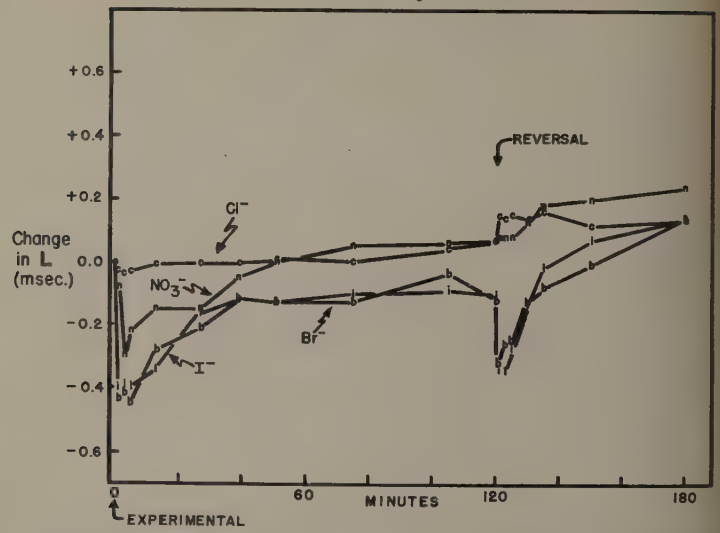


FIGURE 9. Effects of anions on the time to the end of the latency relaxation. Procedural details as in FIGURE 2. Average deviation of all points, ± 0.04 msec.

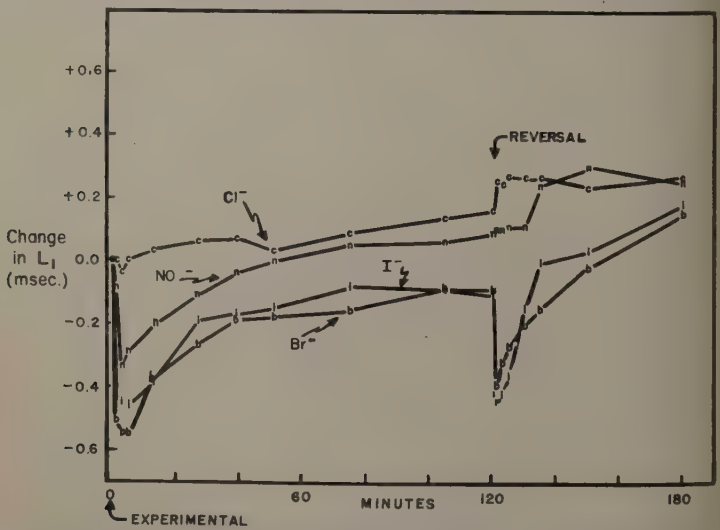


FIGURE 10. Effects of anions on the time to the onset of positive tension development. Procedural details as in FIGURE 2. Average deviation of all points, ± 0.05 msec.

atency of the latency relaxation, and of L and L_1, which together neasure the latency for positive tension output, has been previously ound in other work on the latent period dealing with effects of initial nuscle tension,[14] of previous activity in both normal[19] and in iodoacetate oisoned muscle,[20] and, to some extent, of pH.[17] All these lines of evidence thus combine to indicate that, in addition to the overt mechanical differences of a stimulated muscle during the various portions of the latent period, a process is underway in the muscle during the interval L_R, which is qualitatively distinct from that proceeding within the period L_1 to L_R.

In addition to the above changes in the latency time parameters, it was also found that the depth of the latency relaxation, R, was altered (FIGURE 11). The results show that both the NO_3- and I-anions cause moderately large reversible increases in R, whose time course is roughly like that described earlier for the variations in T (FIGURE 2). The corresponding values for the Br-treated muscle are not included in FIGURE 11 since, for some unaccountable reason, these were too variable for analysis. In so far as the plotted results go, however, it is evident that the anions show relative effectiveness in determining the value of R in the order $Cl < NO_3 < I$.

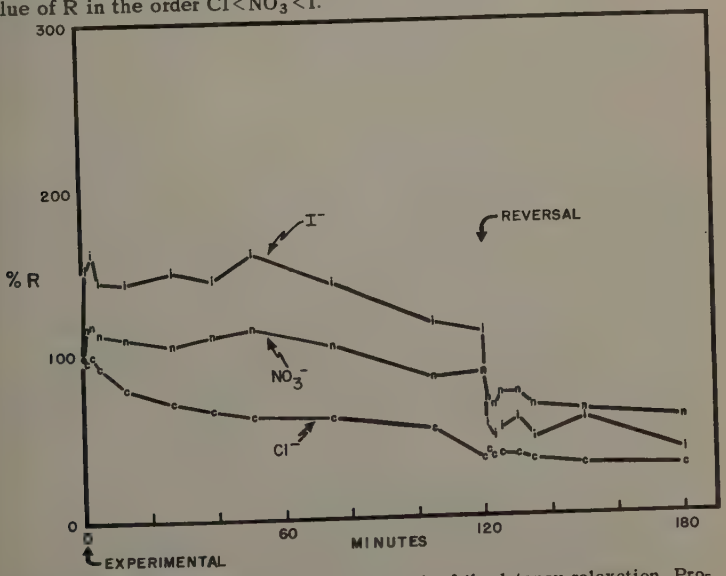


FIGURE 11. Effects of anions on the depth of the latency relaxation. Procedural details as in FIGURE 2. Average deviations: Cl, ± 16 per cent; NO_3, ± 14 per cent; I, ± 24 per cent.

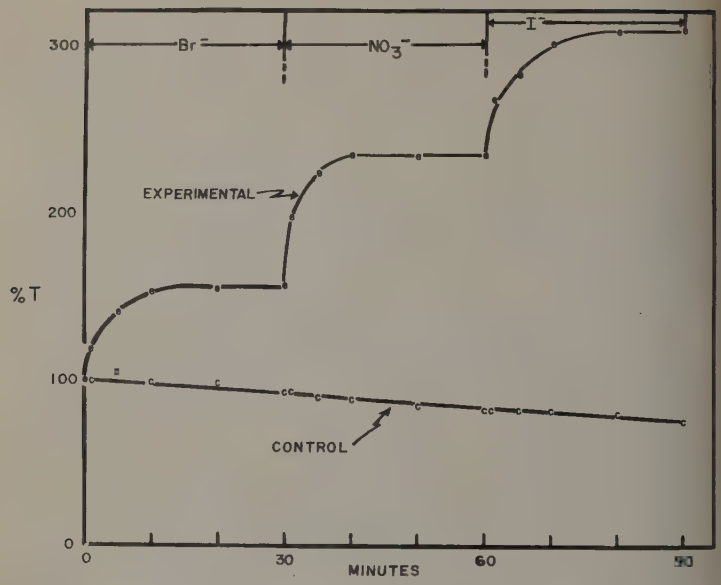


FIGURE 12. Increase in peak tension of isometric twitches of muscles successively exposed to Br, NO_3, and I-Ringer. Average deviation of all points, ± 16 per cent.

(2) *Isometric twitch responses of sartorii treated with a series of experimental anions.* Following equilibration and mounting of the sartorius in the massive electrode chamber, two normal maximal responses were obtained as in the preceding section. The chloride-Ringer was then removed and replaced with bromide-Ringer, and twitch responses were recorded at 1, 5, 10, 20, and 30 minutes after contact with the experimental solution. After 30 minutes, the bromide-Ringer was removed and replaced with nitrate-Ringer. Responses were again recorded at 1, 5, 10, 20, and 30 minutes, after which time the nitrate-Ringer was in turn replaced with iodide-Ringer and the same sequence of responses was obtained. Control muscles were subjected to the same treatment with the exception that fresh chloride-Ringer was exchanged in place of each experimental solution.

Not all of the results obtained in this way will be presented, since they uniformly corroborate conclusions drawn from previously given results. In general, however, they show a marked degree of regularity, as is apparent in the smoothness of the curves for the average values of T for all muscles tested, illustrated in FIGURE 12, where this variable

s plotted in the usual way. Among the points of major significance in hese results there are: (1) the rapidity with which each ion was able to effect a potentiation of twitch tension (approximately 85 per cent of the naximal change occurred within the first 5 to 10 minutes after contact); (2) the relative rapidity with which an equilibrium was attained; and (3) the relative magnitudes of the potentiations themselves. The time parameters of the twitch (exclusive of the latent period) were increased in a manner similar to their behavior in previously described results and, as in the previous experimental group, the three experimental anions exerted equal effects in their ability to speed up the latent period events.

(3) *Action potentials accompanying the twitch responses of nitrate-treated sartorii.* Sartorii used in this series were curarized (0.0005 per cent d-tubocurarine chloride*) during equilibration and throughout the experimental treatment, but results obtained with directly stimulated, noncurarized sartorii differed in no apparent manner from those obtained using curarized preparations. The muscles were affixed to the isometric lever and the peak tension developed in each maximal twitch response was recorded along with the corresponding action potential. Two records of the pretreatment maximal responses were obtained for each muscle. These were separated by a two-minute interval. In the first group of experiments, the sartorii were then placed in 100 ml. of nitrate-Ringer in a beaker and oxygenated for four minutes, after which they were remounted in the moist-chamber. They were stimulated with the same previously slightly supermaximal stimuli and the action potentials and the peak tensions were recorded. They then were returned to the experimental solution until one minute prior to the next observation. Records were obtained at 5, 13, 27, 39, 75, and 120 minutes. In the second experimental group, the recording of the normal maximal responses was followed by a determination of the shock strengths necessary for a 50 per cent reduction of both the electrical and mechanical responses, and records of two such responses were obtained. The muscles were treated as in the preceding experimental group except that the records were obtained at 10, 30, 60, and 120 minutes for those shock strengths that were maximal prior to the experimental treatment and for those shock strengths that had produced 50 per cent maximal response prior to the experimental treatment (similar experiments have been performed with either Br or I, the experimental anion, instead of NO_3, but these yielded results exactly like those with NO_3, and so need not be presented here in detail).

The results of this series further substantiate the conclusion that the potentiated tension outputs of sartorii treated with these experimental anions are not the result of the excitation of fibers that were previously

*It is a pleasure to express our thanks to E. R. Squibb and Sons, New York, N. Y., for generously supplying us with the d-tubocurarine chloride.

unresponsive. Whereas nitrate enabled the previously normal 50 per cent maximal stimulus to elicit a maximal action potential, it showed no ability to alter the electrical response to stimulation which was maximal prior to treatment. FIGURE 13A shows a typical record of the diphasic

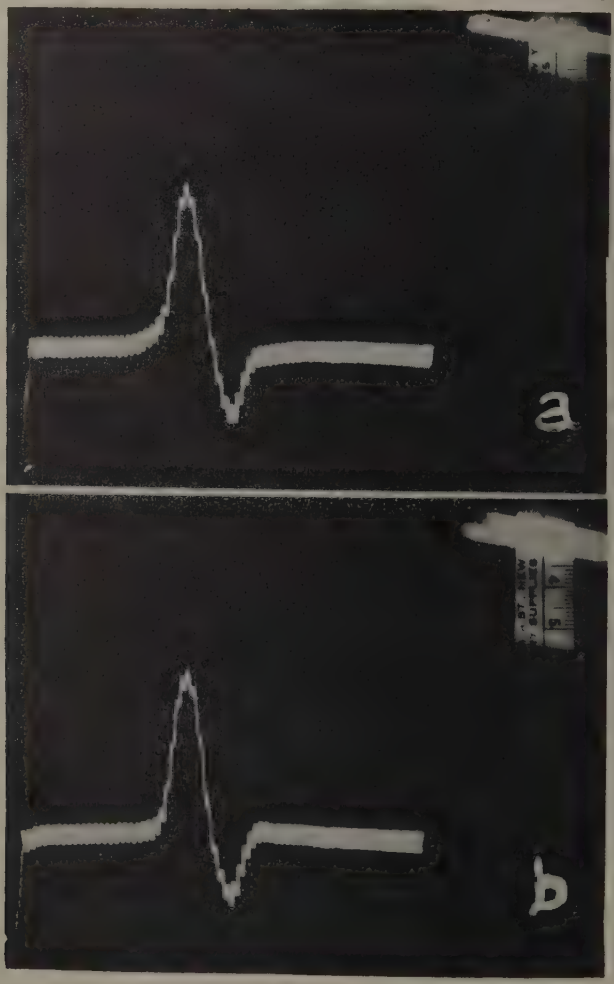


FIGURE 13. Records of action potentials and peak tensions of maximal twitches of a sartorius muscle A, in Cl-Ringer, and B, after 30 minutes immersion in NO_3-Ringer.

TABLE 1

Time in NO_3-Ringer, minutes	Response to stimulus held at original strength of 50% maximal		Response to stimulus initially of maximal strength
	A, %	T, %	T, %
0	50	50	100
10	95	202	214
30	97	208	216
60	100	233	213
120	100	185	188

The average per cent variations in the height of the first limb of the diphasic action potential (A) and of the peak twitch tension (T) developed by sartorii in response to 50 per cent maximal and maximal stimulation after treatment with NO_3-Ringer. The normal (pretreatment) maximal values of A and T are each set at 100 per cent.

Average deviation for A values, ± 17 per cent.
Average deviation for T values, ± 7 per cent.

action potential and the peak isometric tension obtained by the direct, slightly supermaximal stimulation of a curarized normal sartorius. FIGURE 13B indicates the action potential and peak mechanical change for the same muscle in response to the same stimulation 30 minutes after contact with nitrate-Ringer.* Although the twitch tension had been increased about 300 per cent by the action of nitrate (which, though much higher than usual, does occasionally occur), the action potential remained unchanged in both amplitude and timing. This result is of great importance, for it proves that the electrical response is made up of only a single action potential, so that, even though the mechanical responses of the anionically treated muscle were so highly potentiated, these were nevertheless twitches. This is in contrast to the augmented type of mechanical output of, *e.g.*, veratrinized muscles stimulated with a single shock, for here the presence of repetitive action potentials proves that such enhanced responses are tetani and that, accordingly, this in itself can explain the augmentation in mechanical output without need of assuming increased strength of inherent contractility of the fibers. Further, it is especially to be noted (see TABLE 1) with respect to the tension outputs of nitrate-treated muscles stimulated with the previously submaximal shocks that, not only were maximal responses obtained, but the peak isometric tension outputs of such muscles were equal to those obtained by stimulating the same muscle with the previously 100 per cent maximal stimuli. This is to say, the nitrate (and

*This record has already been published.[13] It is again included here in order to document further the present discussion.

the same can be said for the bromide or the iodide) acted in a dual manner; (1) to increase the excitability of the fibers, thus making the previously 50 per cent maximal stimulus an at least maximal stimulus; and (2) to increase the maximal twitch tension output of the individual muscle fibers.

From all the foregoing, it is evident that the three anions we have used all caused essentially similar qualitative alterations in any one parameter of our study. It did not appear to be of interest, at this time, to search for additional evidence of quantitative differences due to the different anions. Hence, in the following, the experiments are concerned with further effects of only the NO_3-ion, this anion having been chosen since its effects were much more pronounced than those of Br, and were more uniform than those of I (see, *e.g.*, FIGURES 6 and 7).

(4) *Twitch tension as a function of NO_3 concentration.* Experiments in this series were done to determine the relation between the potentiation of peak twitch tension and the relative amount of NO_3 in the medium. Following equilibration of the muscle to Cl-Ringer, two normal, maximal twitch responses were recorded. The Cl-Ringer was then removed and replaced with a mixture containing 90 per cent of Cl-Ringer and 10 per cent of NO_3-Ringer. After 10 minutes in this medium, two maximal twitch responses were again recorded. This procedure was then repeated at 10 min. intervals for mixtures of the two types of Ringer which varied in 10 per cent steps, until finally the medium consisted only of NO_3-Ringer.

The results, plotted in FIGURE 14, prove that maximal potentiation was obtained in the pure NO_3-Ringer. Yet it is noteworthy that a mixture containing only 10 per cent of the NO_3-Ringer led to a potentiation which was practically 40 per cent as great as that caused by the 100 per cent NO_3-medium.

(5) *Isometric twitch responses of sartorii treated with nitrate-Ringer and subjected to activity.* Having ascertained that these anions could bring about and maintain a considerable potentiation of the isometric tension developed by these muscles, it was of interest to consider the question of the ability of such treated muscles to maintain this increased mechanical output within a period of concentrated activity.

Sartorii were massively stimulated with slightly supermaximal shocks, and two normal isometric twitch responses were recorded. The chloride-Ringer was removed and replaced with nitrate-Ringer, and twitch responses were obtained at 1, 5, 10, 20, and 30 minutes thereafter. Beginning with the response at the 30th minute of contact with nitrate-Ringer, the muscles were subjected to an activity series (12 to 18 minutes in duration) at the rate of one slightly supermaximal stimulus every 1.5 seconds (40 per minute). Control muscles were treated similar-

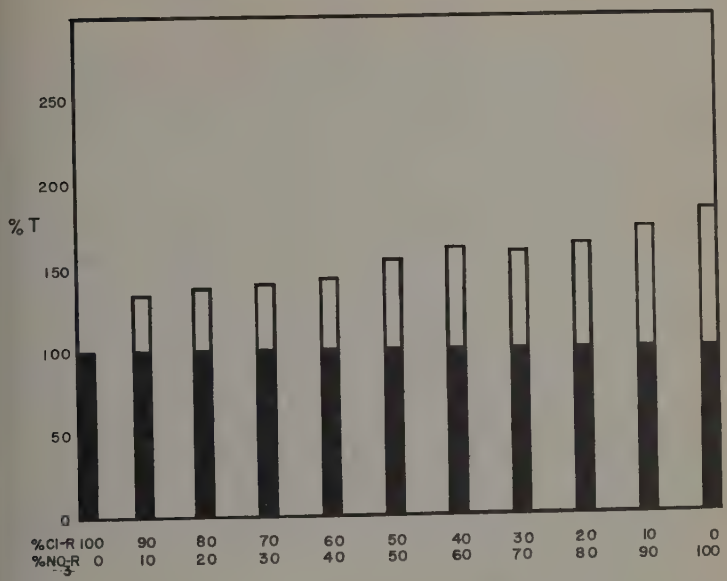


FIGURE 14. Increase in peak tension of maximal twitches of a muscle immersed in mixtures of Cl- and NO_3-Ringer. The period of exposure to each mixture was 10 minutes, and the muscle was exposed successively to media of greater relative content of NO_3-Ringer. Average deviation for all experimental changes, ± 12 per cent.

ly, but these muscles were in contact only with Cl-Ringer. A third group of muscles served as an additional control. These latter were stimulated with two slightly supermaximal stimuli separated by 1/50 of a second. Each two such shocks were considered as one stimulus throughout the treatment, including the activity series.

The reduction of tension output in activity must, of course, be compared with a fatigue curve obtained from similarly-treated normal muscles in contact only with chloride-Ringer. A second type of control, however, was found to be necessary. The nitrate-treated muscles developed maximal twitch tension, which was generally of the order of two times normal. If normal muscles (in chloride-Ringer) are given two maximal stimuli separated by an interval of 1/50 of a second (at 25.0° C.), the mechanical output will be approximately two times the normal value. The muscle responses, while not simple twitches, nevertheless correspond to the nitrate-treated muscles in at least one characteristic, namely, they develop summated tensions in response to two such stimuli, which are approximately equal to those developed by nitrate-treated

muscles stimulated by single maximal shocks. We shall refer to such Cl-contractions as simulated NO_3-responses.

The results for the three groups of activity series are plotted in FIGURE 15 as the mean percentage tension changes in activity as a function of the time after the start of activity. The mean peak isometric tension for each group before the start of activity was taken as 100 per cent. It is obvious that the nitrate-treated muscles became fatigued in approximately one half the time required for an equal fall in the tension outputs of the simple-twitch control muscles. Simulated nitrate-treatment, however, resulted in activity series that were quite like those obtained for nitrate-treated muscles. It is possible, therefore, to conclude that the more rapid fatigability of NO_3-treated muscles was not due to some direct action of NO_3 on fatigability *per se*, but resulted rather, indirectly, through the limited capacity of the isolated muscle to maintain a higher than normal degree of mechanical output in continuous activity. Thus it appears that while nitrate treatment permits the muscles to develop greater than normal tensions in single twitches, nevertheless the nitrate-treated muscles do not produce a total amount of mechanical output

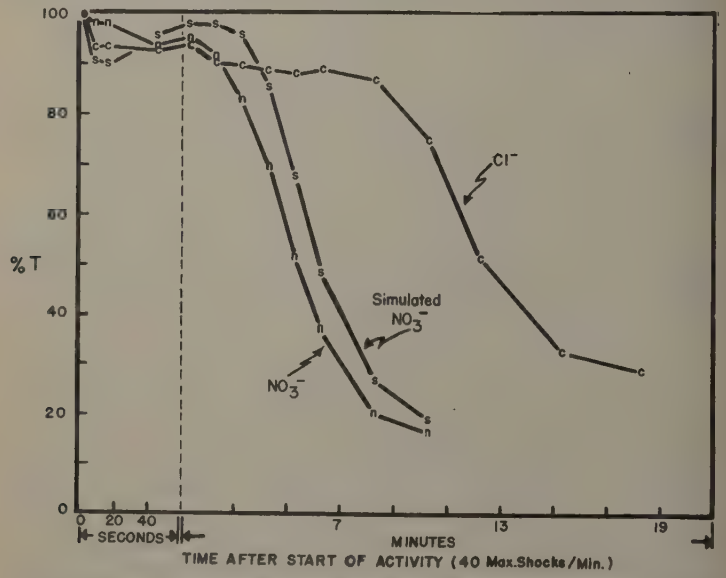


FIGURE 15. Effect of activity on isometric output of muscles in Cl- and in NO_3-Ringer, and in Cl-Ringer under conditions of stimulation to simulate the tension potentiation of NO_3 treated muscles. See text for further details. Average deviations: Cl, ± 17 per cent; NO_3, ± 6 per cent; simulated nitrate, ± 6 per cent.

greater than that obtainable from normal muscles; nor, for that matter, did such treatment decrease this output. Thus, these experiments indicate, in general, that the NO_3-treatment leads to manifest effects on contractile output of the muscle, as such, but not to any effect connected with fatigability of contractile behavior.

(6) *Strength-duration relationship for nitrate-treated sartorii.* Sartorii in this series were curarized (0.0005 per cent d-tubocurarine chloride) throughout the equilibration and experimental periods, and the action potentials of these muscles were used as threshold criteria. No photographic records were obtained, but the minimum voltage required to elicit a just noticeable response at a given duration of stimulating pulse was observed and recorded. Stimuli were rectangular-wave shocks at durations of 10, 20, 25, 30, 45, 60, 110, 120, and 380 microseconds. Following these determinations for equilibrated muscles, such muscles were then immersed in oxygenated nitrate-Ringer for 30 minutes, after which they were remounted in the chamber and the threshold shock strengths were then redetermined for each of the above durations. Control muscles were similarly treated, except that these latter were in contact only with chloride-Ringer throughout the duration of the experiment. It should be noted that this method provides a test for excitability of only the most excitable fibers of the muscle. The results of this series of experiments prove that there were no significant changes in the strength-duration relation for sartorii treated with nitrate-Ringer. Thus, while the results obtained with 50 per cent maximal stimulation indicated a considerable increase due to NO_3 in the excitability of the less excitable of the muscle fibers, the strength-duration determinations indicate no definite change in the excitability of the most excitable fibers.*

(7) *Latent addition in nitrate-treated sartorii.* Experiments were arranged in which conditioning shocks of 90 per cent threshold value were applied to the muscles. These shocks were followed at various intervals by a second shock of sufficient strength just to cause excitation. The fraction of threshold voltages necessary for this second

*It may be mentioned that, in our finding, the fact that NO_3 does not increase the excitability of the most excitable fibers of the frog sartorius is in contradiction with Chao's[12] result indicating the presence of such an increase. This disparity may be due to the following technical differences: (1) Chao used the kymographically recorded mechanical response of his sartorii to indicate threshold excitation, while we employed the much more sensitive method of action-potential recording under high amplification; (2) Chao's results were obtained from two different groups of muscles, one serving as control, and the other as experimental material, but our data were obtained from experiments each involving one muscle tested first in the control, and then in the experimental medium; and finally (3) Chao's experimental solution (since it consisted, *e.g.*, of 40 per cent of isotonic $NaNO_3$ and 60 per cent of Cl-Ringer) varied not only the anion content but also the ratio of Na to each of the other cations (K and Ca) while, in our work, the NO_3 completely replaced the Cl without any change in cation composition. Despite these differences, however, our results do agree with Chao's with respect to the threshold reduction of the less excitable fibers of the sartorius; and, taken as a whole, our data indicate that the effect of NO_3 is to narrow the range of excitability of the fibers of the whole muscle.

or test shock was an indication of the reduction in threshold brought about by the conditioning stimulus. The action potential was used as the criterion for the visual determination of threshold. In one group of experiments, rectangular-wave pulses of 100 microseconds' duration were used for both the conditioning stimuli and the test shocks. In a second group, both types of stimuli were set at 20 microseconds' duration. Threshold voltage was determined for each sartorius and the voltage was then reduced to a value that was 90 per cent of threshold. This pulse was then used as the conditioning shock (S_c). Threshold voltage was then determined for the test shock (S_t) at 10, 30, 50, 100, 200, 300, 500, and 750 microsecond intervals between S_c and S_t followed, at each of the durations, by a redetermination of the threshold voltages for both S_c and S_t when used singly (this insured against a change in threshold voltage due to a possible change of the pulse duration). The muscles were then placed in oxygenated nitrate-Ringer for 30 minutes, after which time the above procedure was repeated.

$$\text{The ratio, } \frac{\text{threshold voltage} - \text{test shock voltage}}{\text{Threshold voltage}}, \textit{ i.e., } \frac{\text{Th} - E_{S_t}}{\text{Th}},$$

(after Katz[21]) was used as a measure of the conditioning effect of the first shock. The results of this series were much the same for both

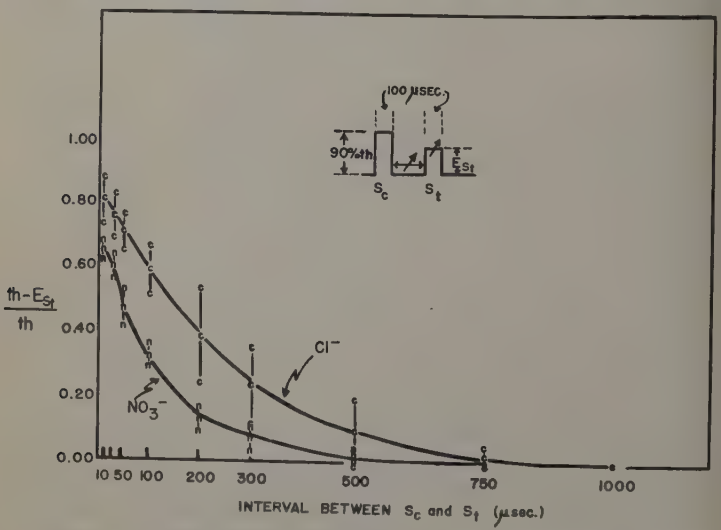


FIGURE 16. Latent addition of the local excitatory disturbance of muscles in Cl- and in NO_3-Ringer. See text for further details.

durations of stimulating shocks. A graph of the mean values (100 microsecond durations) obtained for normal sartorii and for the same muscles, after 30 minutes of immersion in nitrate-Ringer's solution, is shown in FIGURE 16. It is apparent that the effect of the nitrate ion was such as to reduce the time constant for the decay of the local excitatory state.

(8) *Miscellaneous observations*. The effect of NO_3 on the maximal isometric tetanus output was first studied by subjecting a muscle to a 0.1 sec. tetanus stimulus at a frequency of 50/sec. initially in Cl-Ringer and then, after immersion for one hour in NO_3-Ringer. Under these conditions, the average tetanus tension of the NO_3-muscle was about 25 per cent higher (average deviation ± 11 per cent) than that of the Cl-muscle.[22] The derivative records for both types of muscle, however, clearly indicated that the tetanus output was not perfectly fused, and that the NO_3-muscle exhibited better fusion than the chloride treated tissue. In later experiments (details of which will be presented elsewhere), care has been taken to insure that all tetani were perfectly fused and, under these conditions, the NO_3 muscle shows no potentiation of the tetanus.

A few studies have been made with isotonic responses, and it has been shown that twitch shortening is greatly potentiated by NO_3-treatment.*

Discussion

It is evident from our work that Br, NO_3, and I, each serving as anion instead of the usual chloride in an otherwise normal Ringer's solution, very markedly alter many features of the responsiveness of amphibian skeletal muscle fibers. Some of these changes appear as increases in certain excitability parameters, as in excitability itself as indicated by the lowering of threshold of the less excitable fibers of a muscle, and in rapidity of decay of the local excitatory disturbance. Our findings concerning the lowering of threshold are, in essence, confirmatory of earlier studies of the effects of the above anions, in which, among other tests for excitability changes, it was found that greater mechanical responses were obtained in the presence of these anions than in ordinary Cl-Ringers, when muscles were stimulated by various nonelectrical agents,[5,8,9,10] or by originally submaximal electric shocks.[11,12] It should be emphasized that in general, in this earlier work and, especially, in Chao's study,[11] any increase in contractile output of a muscle was attributed solely to recruitment of fibers, which was made possible by the increase in excitability engendered by the experimental anion.

*It may be further noted that Elsie Siegel, in her thesis for the degree of Master of Arts (Graduate School of Arts and Science, New York University), finds that in isotonic contractions of the frog sartorius, the shortening in Ringer's containing the various anions is in the order $Cl < Br < NO_3 < I$, but the diphasic action potential is not modified by any of the experimental anions.

Evidently unnoticed in previous studies, but stressed in ours, however, is the fact that the anions of interest, in addition to their excitability effects, can act to alter profoundly the contractile behavior of the individual fibers of a muscle. As discussed fully in describing our results, we have eliminated recruitment of fibers as a basis for alteration of the experimental mechanical responses by insuring, through use of supermaximal shocks, that the control responses in Cl-Ringer were maximal. Furthermore, our electrical recordings, which demonstrated only a single action potential in each response of the treated muscle to a single shock, indicate that such responses are not involved with summated effects of repetitive excitations, but are simple twitches. Thus, our results, though obtained on whole muscles, prove that each of the constituent fibers, under the influence of our anionically modified Ringer's solutions, produce, in response to a single shock of adequate strength, an isometric twitch that, in comparison with the twitch in Cl-Ringer, shows (1) a hastening of the latent period events; (2) an increase in depth of latency relaxation; (3) a general prolongation of the various subdivisions of the time-course of the twitch; (4) an increase in rate of both contraction and relaxation; and (5) a large potentiation of the peak tension that, when fully developed in I-Ringer, and even occasionally in NO_3-Ringer, results in a twitch tension almost equal to full tetanus output.

Although slightly supermaximal shocks were necessary to insure maximal responses of the chloride-control muscles, it is noteworthy that, despite our use in general of shocks of the same electrical strength for the treated muscles, such high-shock intensity was not really needed to evoke maximal responses in these muscles. For the recruitment effected by increase in excitability due to the anions enabled us to elicit maximal responses from, *e.g.*, the NO_3-treated muscles, even when they were activated by shock strengths only 50 per cent of maximal for Cl-muscles. Yet, in such responses, there was evident all the characteristic effects of the anions. This result is significant, for it proves that, even though many of our experiments involved stimulation of the treated muscles by the same shock strength used for the Cl-muscles, the resultant great increase in physiologically supermaximal intensity had nothing to do with the production by these muscles of the effects of the anions.

Although the twitch potentiation is the most striking effect of these anions, it is noteworthy that the accompanying increases in the time parameters of the contraction and relaxation periods are themselves independently indicative of changes in the intrinsic contractile behavior of each fiber. For, if the anionic effects were manifest only as excitability increases, the resultant recruitment of fibers would, if anything, tend to shorten the twitch time (by decreasing the amount of inactive tissue in

the activated muscle), whereas what we observe is the opposite of this. Furthermore, all these changes evidently involve alterations only in mechanical behavior as such, and not of the metabolic systems concerned in the maintenance of mechanical output during continued activity—this being indicated by our experiments, which demonstrated that, for example, NO_3-treated muscles fatigue at the same rate as do Cl-muscles that had been activated by dual-shock stimuli so as to simulate the potentiated NO_3-response.

In attempting to explain the mechanism of production of the anionic effects, it is necessary first to note that the muscle fiber membrane is permeable to these ions [23, 24] and that, therefore, it is conceivable that their direct action might occur on any part of the fiber, *i.e.*, on the internal contractile system, as well as on the superficially located membrane system. Our results, however, indicate that all the observed effects are consequences of a direct action on the fiber membranes. This is self-evident, of course, for obvious membrane effects, among which are not only those already mentioned in our work (increase in excitability of fibers as expressed in decrease in threshold and in increased rate of decay of the local excitatory state), but also those changes observed by others, in resting potential [25, 26, 27, 28] and in permeability.[23] It is evident that the membrane is sensitive in a variety of ways to the action of the anions of interest, yet it is to be noted that our results indicate they do not affect the magnitude of the diphasic spike potential, and this suggests that these anions do not alter the Na-mechanism which is responsible for generation of this potential.[29]

Turning now to the contractile changes: analysis of certain kinetic features of our results in relation to the rates of movement of the anions into the muscles substantiates our view that here also we are dealing with consequences of a direct action of the anions on the membrane. Passage of the ions from an external medium into the muscle depends on two different transport mechanisms: first, diffusion into the interstitial muscle space; and, second, penetration into the intrafibrillar space. It is evident that the former transport process will bring the anions into contact with the fiber membranes, and that the latter must occur if the anions are to make contact with the material of the contractile system. Hence it is clear that, to prove our view that the changes in contractile behavior result from membrane effects of the anions, it is at least necessary for us to demonstrate that the kinetics of change of the mechanical parameters, as the muscles are exposed to or removed from the experimental anionic media, are more in accord with changes in concentration of the anions interstitially than with such changes within the fibers.

In respect to the diffusion process, we first note that this involves

essentially the sodium salts of the various anions, since the corresponding K and Ca salts of the various modified Ringer's solutions are in such low concentrations that we can safely neglect their participation in the process. Now it has been shown[30] that KCl passes into the frog sartorius' interstitial space with a diffusion constant only one tenth of that for free diffusion in water. We assume that the same relation holds for the Na salts of our anions. According to *International Critical Tables* (vol. 5, p. 67), the diffusion constants of these substances in water at about 15° C. are all close to 6.0×10^{-4} cm.^{2}/min. Using a Q_{10} of 1.33 to correct for our experimental temperature of 25° C., and taking into account the 10-fold reduction factor mentioned above, we have for the diffusion constant (k) of these substances in the muscle's interstitial space a value of 0.80×10^{-4} cm.^{2}/min. What this means concerning accumulation of the anions in the interstitial space can be seen by using Hill's theory[31] (for the case relevant to our problem, *i.e.*, two-sided diffusion into a plane sheet) to determine as a function of time, after immersion of a muscle in one of our experimental solutions, the average degree of saturation ($\bar{c}$) of the interstitial muscle space in respect to the concentration of an anion in the external medium. To do this, we first calculate the quantity kt/b^2, in which t = time in minutes, and $2b$ = thickness of the frog sartorius, which we here take equal to 0.07 cm.; and then determine $\bar{c}$ from Hill's figure 5. We thus find, *e.g.*, for $t = 5$ min., $\bar{c} = 0.64$; for $t = 10$ min., $\bar{c} = 0.84$; and for $t = 15$ min., $\bar{c} = 0.93$. An alternative calculation is of interest: if we use Hill's figure 4 and make the necessary adjustments relevant to our problem, it can be shown that, after an elapsed time of 27.6 min., the concentration of an anion at a level midway through the thickness of a muscle will be 0.95 of that in the external medium, while the concentration at any other level will, of course, be closer to that of the medium. Hence, even at 15 min. the interstitial space of the muscle is rather well saturated with the anion and, at 30 min., it is for all practical purposes fully saturated. Furthermore, the same data may be used, but in reverse, so to speak, to account for the loss of anion from the fully saturated extracellular space when the muscle is replaced in the normal, Cl-Ringer's solution. So that, for example, such a muscle should have its extracellular space practically free of an experimental anion some 30 minutes following replacement in Cl-Ringer.

As for the second transport mechanism, penetration into the fibers, this is, at best, a much slower process: the work of Conway and Moore[24] shows that Br and NO_3, as K salts, penetrate the muscle membrane, but about 200 minutes are required for the fibers to come into equilibrium with the external concentration (0.1M) of these salts. These authors did not study KI, but we shall make the reasonable assumption in the

following that this penetrates at about the same rate as KBr or KNO_3. In our case, however, the anions were not present as K salts to any meaningful extent, but rather as Na salts, and these should not have penetrated at all since, under Conway and Moore's conditions, which hold essentially also for our experiments, such penetration would have been prevented by the inability of the Na ion to pass across the membrane. Nevertheless, it should be possible for the anions, as such, to enter the fibers by exchange with internal chloride. This would occur at about the same slow rate as the penetration of the corresponding K salts.[23] But since the intracellular concentration of Cl is, at most, 0.01M,[32] the electrochemical conditions for equilibrium would set this concentration as an upper limit for the internal accumulation of an exchangeable anion, even though the external concentration (as held generally in our experiments) was 0.115M.

We shall now compare with this evidence concerning transport of anions the temporal features of development or disappearance of the various contractile alterations of muscles following, respectively, their exposure to, or removal from, the experimental media. In FIGURES 2, 6, and 7, we note that the value of practically all the different parameters increased during the experimental period to essentially maximal values in times of about 15 min. or less. Some of these changes developed with exceeding rapidity, *e.g.*, the rise in peak value of twitch tension following immersion in I-Ringer (FIGURE 2), which reached 86 per cent of its final increment in one minute. The effect of the NO_3 on tension output (FIGURE 2) did not plateau until some 90 minutes had passed, yet only 5 minutes was required to achieve 65 per cent of the final total change and at 30 minutes the corresponding figure is 83 per cent. FIGURES 3, 4, and 5 demonstrate that the increase induced by Br in all cases reached their maxima in 20 minutes or less; and FIGURES 8 through 11 show that the initial alterations induced by all the anions in each of the latent period parameters became maximal in times of about 5 minutes or less. Thus, in all the cases so far mentioned, the time generally required for production of either the total change in the mechanical parameters or a large fraction of it, is much shorter (by a factor of 10 to 100) than the time required for an equivalent amount of penetration of the anions into the fibers, but it is of the same order that is needed for development in the extracellular space of a comparable degree of saturation with the experimental anions of the external media.

It is true that some of our results, *i.e.*, the modifications effected by NO_3 and I that are presented in FIGURES 3, 4, and 5, seem not to be in agreement with this conclusion. Calculation, however, shows that, for these, the average degree of change of all the parameters relative to their plateau values reached in the NO_3 and I media is 49 per cent at

30 minutes, and 87 per cent at 60 minutes. It is apparent that even these relatively slowly developed alterations appeared much faster than could be expected if their production had to depend on the very slow penetration of the anions into the fibers.

Turning now to the reversal changes, we see from the results of FIGURES 2 through 7 and of FIGURE 11, that, without exception, each reversal is essentially completed in about 30 minutes, and that a major part (approximately 80 to 90 per cent) of each is attained in 2 or 3 minutes. It is especially noteworthy that this behavior obtains even for those parameters for which the initial effects due to NO_3 and I (FIGURES 3, 4, and 5) developed rather slowly. Similarly rapid changes occur in the experiments of FIGURES 9 and 10, if we consider at least the initial reversal events. It is clear from all these results that the kinetics of these reversals is much too fast to be related to the very slow exit of anions from the intrafibrillar space, but that it is in excellent correlation with the speed by which the diffusion process would remove the anions from the extracellular space of the muscles.

Now, all the preceding evidence indicates that the presence of the experimental anions in the extracellular space—and therefore in contact with at least the outer boundary of each fiber membrane—provides a sufficient condition for production by the muscles of the various mechanical effects observed in our experiments. We therefore infer that the anions exert these effects by a direct action on the cell membranes, which is then somehow "relayed" into the interior of the fibers and thus becomes manifest indirectly as the observed changes in behavior of the contractile system. Presumptive evidence in further support of this inference is indicated in the following. It is certain that the state of the contractile system, in general, is dependent on the state of the membrane, as is shown in the mechanical changes engendered by transmembrane potential variations in both contracture and normal excitation-contraction (*i.e.*, E-C) coupling.[33] Hence, it is conceivable that membrane alterations that have been induced—as by our anions—in the resting muscle fiber might affect the E-C coupling mechanism so as to lead to changes in contractile response of the excited fiber. A case of this sort has already been demonstrated by us in earlier work,[3] in which it was shown that, under certain conditions, the K ion modifies the mechanical behavior during both latent and contraction periods of the twitch, even though it acts directly only at the membrane. It is also noteworthy that Hayashi[34] has demonstrated that actomyosin threads, formed by his method from initially surface-spread films of the protein, show no difference in mechanical action when the prevailing anion of his media is changed from chloride to nitrate. This finding suggests that, even if the nitrate in our experiments had penetrated into the

bers, it would have had no observable effect on the mechanical activity the actomyosin of the living contractile material.

In attempting to account in detail for the proposed action of the nions, we note, first, that there is no doubt that these agents do affect ie membrane, as is evident in our previous discussion concerning hanges in excitability, electric potential, and permeability. It is known rom earlier work, especially on excitability, that anions exert such iembrane effects in a graded fashion in accordance with the usual yotropic series, *i.e.*, $Cl<Br<NO_3<I$. This series has customarily been offered as the order in which these anions affect various properties, *e.g.*, dispersibility of colloidal and, more specifically, of protein systems. The membrane is known to be composed of such systems, and it follows, therefore, that the known changes in membrane function caused by the anions may be due to the action of these agents on the colloidal structure of the membrane. Now, it is evident that, in practically all of our results, the relative effectiveness of the anions in causing changes in mechanical behavior also falls into the lyotropic series. This indicates that even though the anions do not directly influence the function of the contractile system, the intensity of their indirect action is determined by the effectiveness of their primary lyotropic actions on the membrane.

We can only speculate on how such primary effects are relayed inward. Two different, general mechanisms, however, may be postulated. In the first, we suppose that, in the resting fiber, the altered membrane permits introduction of some change in make-up of the contractile system which, when activated, then functions in the altered fashion of our experiments. The intrafibrillar change, for example, might result from release of a membrane component, possibly an ion, or it might occur by movement of some critical substance into or out of the fiber, in consequence of the increase in permeability due to the anions. In the second mechanism, we may hypothesize that the anionic effects do not induce any important change in the internal composition of the resting fiber but that, upon stimulation, they modify the E-C coupling reactions and thus relay their effects to the contractile system. In an earlier paper,[33] it has been shown that E-C coupling is comprised of a sequence of processes: (1) excitation, in which the rise of the spike potential acts as a trigger; (2) the spike-activation (S-A) link, which is in action during the first half of the latent period (*i.e.*, during the interval L_R), which seems to involve a membrane component that, under the action of the spike potential, is directed inward and thus serves to cause (3) activation of the contractile elements, which occurs in synchrony with, and thus may be manifested externally by, the latency relaxation, which then induces (4) contraction with its usual manifestation of either muscular shortening or tension. If the transference of anionic

membrane effects to the contractile system is performed by an alteration of E-C coupling, then this must depend on some phase of the sequence which involves the membrane, *i.e.*, phases (1) and (2). But we can eliminate (1), for our evidence indicates that the action potential is not affected by the anions. This then suggests that it is the S-A link that may be altered. Support for this suggestion is found in our results that all the anions cause a decrease in the interval L_R and an increase in the magnitude of the latency relaxation. Both of these changes could be attributed to an S-A link whose intensity had been heightened and which, therefore, as indicated by the earlier and deeper latency relaxation, not only hastened the onset of activation, but also somehow augmented it.

Quite apart from the problem of the means by which membrane effects due to the anions are relayed inward, and also rather independent of our very speculative attempts to analyze it, we now turn to a consideration of the mechanical changes themselves and the problem of explaining them in terms of known mechanisms of contraction. A review of the relevant results (FIGURES 2 to 7 inclusive) indicates that this is complicated by the differences among the various parameters in respect to the course in which changes occur as a function of time of exposure to the experimental media. On the basis of present evidence, it is impossible to construct any simple hypothesis that would explain all these effects. It is fruitful, however, to consider some general results that are highly uniform, since they are independent of the particular anion and of the time of its action. Thus, in comparison with the normal, the experimentally altered muscles produce twitches that always show (1) greater peak tension; (2) longer times both to reach peak and to run to completion; and (3) greater maximum rates of tension change during both contraction and relaxation periods. In our attempt to explain these alterations, we shall keep foremost in mind the tension potentiation which, especially in the nitrate and iodide media, may raise the twitch tension to almost tetanus value.

In order to interpret these results, it is necessary to discuss at some length Hill's conception of the role of the active state in muscular contraction.[35-39] According to Hill, the normal course of overt tension in the muscle twitch is determined by an interaction between internal activated contractile components and passive elastic components in series with them. As previously indicated, the contractile components begin to undergo activation during the latter half of the latent period and thus develop the capacity to shorten; this very abruptly achieves its full intensity which equals that of maximal tetanus. It is maintained so for a duration equal to about half of the contraction period,

nd then subsides during the remainder of the twitch. But these mechanical effects can become evident at the muscle's ends only through nteraction with the series elastic components. When the activated contractile components shorten, they extend and thus cause tension to appear in the elastic units, and it is this tension at any instant that s recordable as force at an end of the muscle, whatever may be the corresponding tension of the active state. Hill's force-velocity relation hat describes the fundamental dynamics of the contractile components ells us that, at best, the velocity of shortening is low and, in fact, decreases as tension mounts in the elastic structures. Hence, in the isometric twitch, the following occurs: Early in the contraction period, when the active state is at full intensity, but tension in the series elastic structures is still low, shortening of the contractile components and hence mounting external tension proceeds at a relatively high though falling rate. Despite falling of activity during the latter half of the contraction period, the intensity of the active state of the contractile components is still high enough to continue to stretch the only partially extended elastic elements, and thus the tension of the muscle continues to rise, but at a still diminishing rate. This dynamic relation goes on until twitch peak, at which moment the tensions of the two types of components are equal to each other and thus also to that of the crest of twitch.[35] Thereafter, as activity still further falls, the highly tensed elastic component is able to stretch the contractile units, and thus ordinary relaxation ensues.

Thus, in an isometric twitch, the active state does not remain at high intensity long enough to transmit its full tension to the series elastic components, and that is why the peak twitch tension is only a fraction of that potentially available in the fully activated contractile components. Although the following quantitative point is not essential for our analysis, it is interesting to note that, in the normal sartorii of our experiments (at 25° C.), the peak tension in twitch is about one third that in tetanus. Since, in respect to the active state, the tetanus tension is a measure of its full intensity, and the twitch tension indicates the residual activity at peak of twitch, it is evident that, during the latter half of the rising phase of the twitch, the contractile components must lose about two thirds of the activity they had when fully activated. Normally, the only way in which a muscle can be made to exert a force equal to that of the fully active state is in a tetanus. In such stimulation, each shock of the series reactivates the contractile components and, if the tetanus frequency and duration are sufficiently great, full activity is maintained long enough to set up the full tension in the series elastic structures.

Now, our results prove that the anions increase the twitch tension.

In terms of the foregoing, this could be due to: (1) an increase in the intrinsic intensity of the active state; (2) a decrease in compliance of the series elastic components; or (3) a prolongation of the period of relatively highly maintained activity. If the first of these possibilities had occurred, this would certainly be signalized by a corresponding augmentation of the peak *tetanus* output, and also, presumably, by no change in the time of rise and fall of the twitch tension. Since our results are not in agreement with these predictions, we can eliminate intensification of the active state as the explanation of the anionically altered twitch output. Consider now the expected effects if there were a reduction in compliance of the series-elastic component. There is no way to alter directly the muscle's internal structure in this way. But Hill's [35] method of applying a quick stretch to a muscle very soon after it has been stimulated, in effect, indirectly accomplishes this; in fact, Hill's conception of the active state is derived from experiments of this sort.[40] And the results of such studies clearly prove that a direct decrease in the series-elastic compliance would act to increase, and advance in time, the maximum of tension developed in the twitch; *i.e.*, the course of twitch tension of such an altered muscle would tend to approach that of its activated contractile component. Now, although our experimentally treated muscles do produce potentiated twitch outputs, the peak of tension appears later and not earlier than the normal. Hence, we conclude that the modifications of the twitch caused by the anions cannot be explained by a decrease in compliance of the series-elastic component.

Of the three possibilities mentioned above, we are now left with the third. If the fully active state, though of normal intensity, is maintained longer, and then decays slower (at least to peak of twitch), than it ordinarily would, there would be provided a longer time for transmission of the tension of the contractile components to the series elastic. There would thus occur a longer contraction period and a greater peak tension before activity becomes so low that relaxation begins. Since our treated muscles do show both these changes, we conclude that a principal effect of the anions is to prolong the period during which the contractile components are maintained at relatively high activity. In corroboration of this mechanism may be mentioned our result (though of a preliminary nature) that the tetanus response to a stimulus of 50 shocks/sec. is better fused in the NO_3-muscle than in the chloride; for the better maintenance, in the former, of tension due to each of the successive shocks is also proof of a longer duration of activity capable of engendering externally evident tension. It should be noted, however, that the mechanism we propose for the anionic effects now being discussed includes two kinds of alteration of the active state: an extension of

ne period of full intensity, and a decrease in rate of the immediately ollowing subsidence of activity. Either or both of these changes could xplain our results, but our present data do not permit us to differentiate etween these possibilities.

Some other features of our results require comment. We have observed hat under the action of the experimental anions the maximal rate of con- raction (D_1) is considerably increased. Since this effect appears quite arly in the contraction period, it may reflect an increase in the rate of setting up the active-state, *i.e.*, of the activation process. In line with his explanation are the previously discussed results that the anions cause a speeding up of the latent period events, but difficult to harmo- nize with this view is our present finding that the time at which D_1 occurs is not changed by the anions. Further work is needed on these effects, and this should be done at a lower temperature than was used for the present experiments, since the resultant slowing of the various processes associated with activation would permit them to be analyzed more precisely.

The alterations of the relaxation period (FIGURES 4, 5, and 7) are also of interest. By comparing the results of FIGURE 5 with those of FIGURE 3, it is noteworthy that, at least for the effects of either NO_3 or I, there is a greater proportionate increase in the time to maximal rate of relaxation than to peak of twitch, for this indicates that the prolongation of activity at a relatively higher level, that accounts for the increased duration of the contraction period, is continuing into the early part of the relaxation period. Thus, these combined effects of more prolonged activity, whatever may happen to the active state in the latter part of the relaxation period, are the major factors that uniquely account for the greater total twitch-time under the action of the experi- mental anions. But it is further noteworthy that a comparison of the changes presented in FIGURES 4 and 5 shows that the total twitch time is proportionately increased considerably less than is the time to maximal relaxation rate. This indicates that the protraction of twitch phases, clearly in evidence up to the early part of the relaxation period, does not continue so as to draw out the later part of this period. This point is also indicated by the results of FIGURE 7, showing that, in general, the maximal rate of relaxation is increased by the anions. Recalling that relaxation is effected when the tensed series elastic elements are able to stretch the weakening contractile structures, it is clear that, under the action of the anions, one factor causing hastening of the later part of relaxation can be attributed to the greater tension established in the series elastic units at the peak of the potentiated twitch. But the results also suggest that another factor in this process is a rather

drastic collapse of the active state rather soon after the crest of twitch is reached.

From the foregoing, it is evident that the altered twitch behavior of muscle under the influence of the Br, NO_3, and I ions, especially the two latter ones, can be explained by their action on the active state. Our interpretation is most definite in respect to the most striking effects of the anions—potentiation of the twitch tension and the associated increase in duration of both the contraction period and the total twitch time—for these are uniquely attributable to the prolongation of the active state. In any case, the changes in mechanical behavior are not dependent on any direct contact of the anions with the contractile material, for, as our work proves, they are indirect consequences of the action of these ions at the fiber membrane. Although our study gives us at least an outline of the changes induced in the behavior of the contractile system, this knowledge contributes nothing to the relief of our practically complete ignorance of the actual membrane changes caused by the anions and the means by which they are coupled to the contractile material. But the results of this paper emphasize the fact—already realized in connection with studies of normal excitation-contraction coupling—that the behavior of the contractile material is sensitively dependent on the state of the membrane, and they present us with some new and highly interesting problems in this fundamental field of muscle physiology.*

Summary

(1) Studies have been made of excitability and of isometric contractions of frog skeletal muscle exposed to Ringer's solutions containing either Br, NO_3, or I as anion in place of the usual chloride. The mechanical changes are recorded by a new dual-channel, piezoelectric, cathode-ray oscillographic method. On one beam of the cathode-ray tube

*It is a pleasure to note that Professor A. V. Hill, following a suggestion by one of us (personal communication by A. S. of October 15, 1951), has measured the twitch heat output of frog sartorii under the influence of the anions[41, 42] and finds that this is considerably increased. The work from Professor Hill's laboratory also includes many types of tests for the effects of the anions on mechanical features of the twitch and tetanus like ours. The results obtained and the conclusions drawn from them in essence confirm those of our present paper. In recent correspondence with Professor Hill concerning this work, he points out that our values of the diffusion constants for the passage of the various anionic salts into muscle are too low. Direct determinations by his collaborators and calculations based on the work of Harris (*J. Physiol.*, **117**: 278-288, 1952) indicate a value for this constant of about 2.3×10^{-4} cm.^{2}/min., instead of the one we have used, 0.8×10^{-4} cm.^{2}/min. Since Harris' work (at least) has been done on sartorii of *Rana temporaria* and ours on sartorii of *R. pipiens*, this discrepancy may be due to a species difference. Even with the greater value of the diffusion constant, however, our analysis, which indicates that only diffusion of the anions to the fiber surfaces is all that is needed to cause the noted effects of the anions on the contractile behavior, still holds. In fact, the extreme rapidity of reversal of the effects of the experimental anions is probably better accounted for on the basis of Hill's larger values of the diffusion constants. Finally, we are greatly indebted to Professor Hill for reading the part of our paper dealing with the active state and for his valuable suggestions for improving our presentation of his conceptions of this state.

s registered a deflection directly corresponding to the muscle's latent eriod tension changes and, on the other, a deflection which gives the ime-derivative of the tension changes of the remainder of the con- raction. Also included in the method are optical myographic means for ecording initial and peak-contraction tensions. Stimulation was effected generally by means of massive shocks, thus facilitating certain pro- cedures and eliminating propagative complications.

(2) After equilibration to any of the modified Ringer's solutions, a muscle, in response to a single supermaximal shock, produces a twitch which, compared to the normal, shows, in general, the following mechanical changes: (a) an increase in peak tension; (b) a prolongation of the contraction and relaxation periods (though in the latter, principally in its early portion), and thus a greater twitch-time; (c) an increase in maximal rate of tension change of both contraction and relaxation periods; (d) an increase in depth of latency relaxation; and (e) a decrease in various durations of the latent period. All these alterations are reversed upon restoration of the muscle to ordinary Ringer's. There is no change in maximal tetanus tension.

(3) The responses to single shocks are proved to be twitches by demonstrating that each involves only a single action potential. The twitch potentiation is not a consequence of fiber recruitment, since control, as well as experimental, responses were maximal.

(4) The relative effectiveness of the experimental anions in producing the above twitch changes is generally in accord with the usual lyotropic series: $Br < NO_3 < I$. The changes due to the NO_3 and I are strikingly large, the typical altered twitches showing, *e.g.*, peak tensions and durations of contraction period of the order of twice their normal values, or, in some muscles, even greater.

(5) When allowance is made for the twitch potentiation in NO_3-treated muscles, it is shown that, in a series of twitches, fatigue develops at about the same rate as the normal.

(6) After the moment of first exposure of a muscle to one of the anion-modified media, the changes in the twitch mount rapidly to their peak values, though at speeds that vary in respect to the particular twitch property. The degree of potentiation of peak tension rises especially quickly and, upon reversal, all the studied twitch parameters revert to normal values with very great rapidity. Analysis of these results in relation to the greatly different speeds with which the anions diffuse into the muscle's interstitial space and penetrate into the fibers shows that the direct action of the anions is only on the surface membranes of the fibers, and that the effects on the contractile behavior develop as indirect consequences of this membrane action. The present work leads to no definite knowledge of the details of these mechanisms,

but certain possibilities are discussed, especially in relation to modifications of excitation-contraction coupling.

(7) Confirming older work, the abnormal anions decrease the threshold for excitation, but only for the less excitable fibers.

(8) The local excitatory disturbance, as tested by the dual-shock technique, decays more rapidly in NO_3-treated muscles.

(9) Analysis based on Hill's conception of the active state proves that the enhancement of the peak tension, the protraction of the contraction period and the total twitch-time are uniquely explained by a prolongation of the active state. Other twitch alterations are discussed in relation to other features of this state.

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